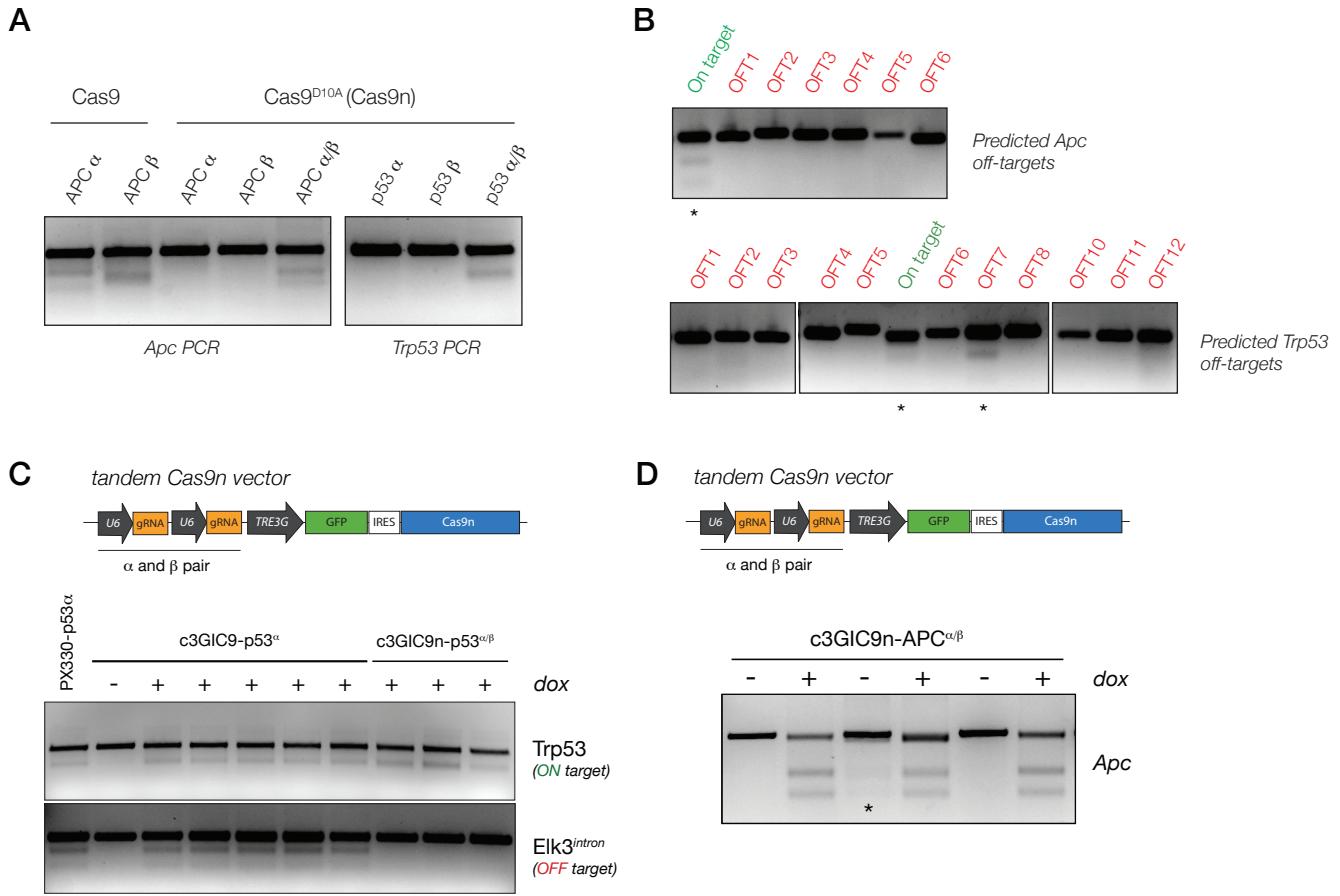
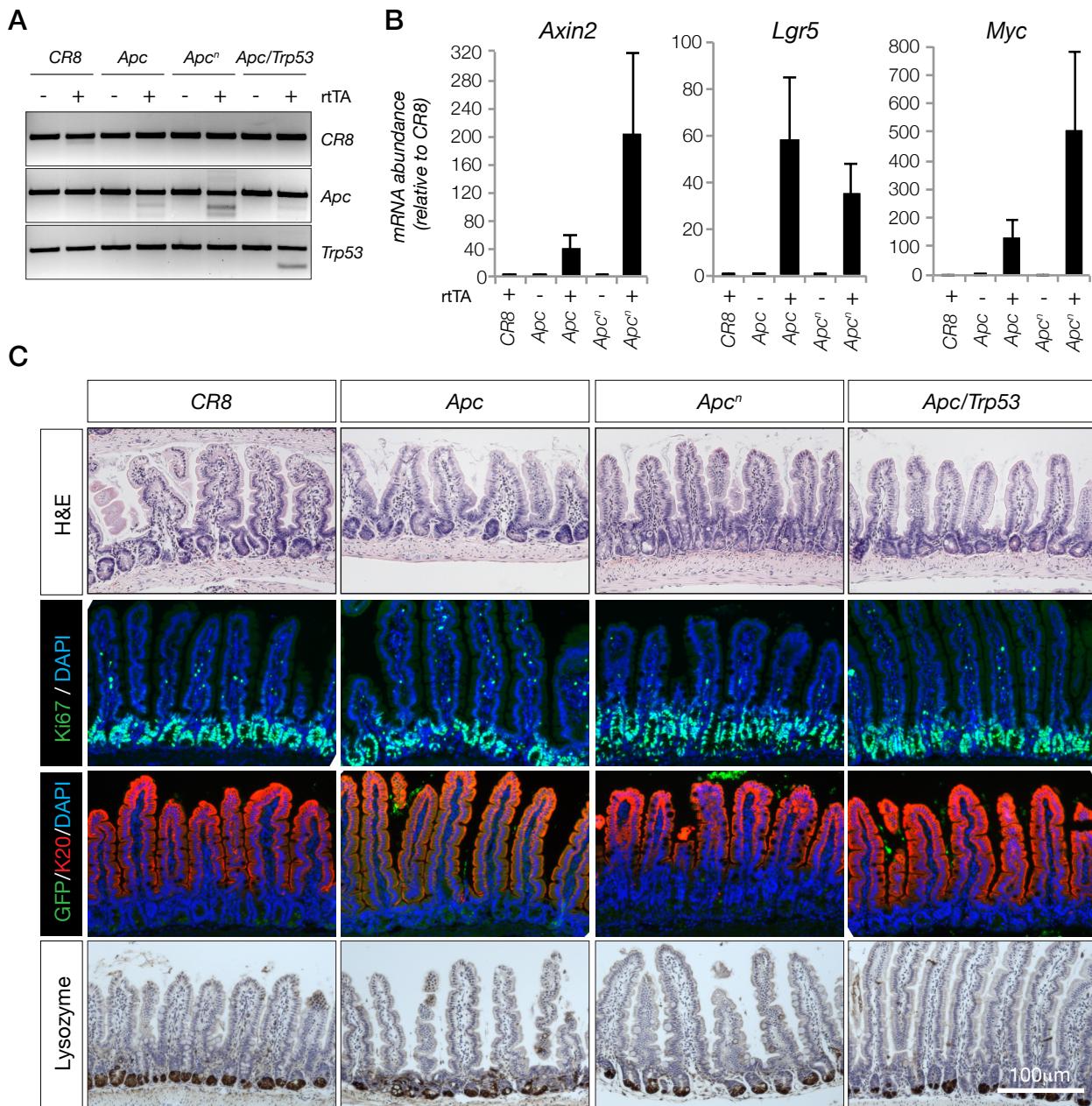


## Supplementary Figure 1



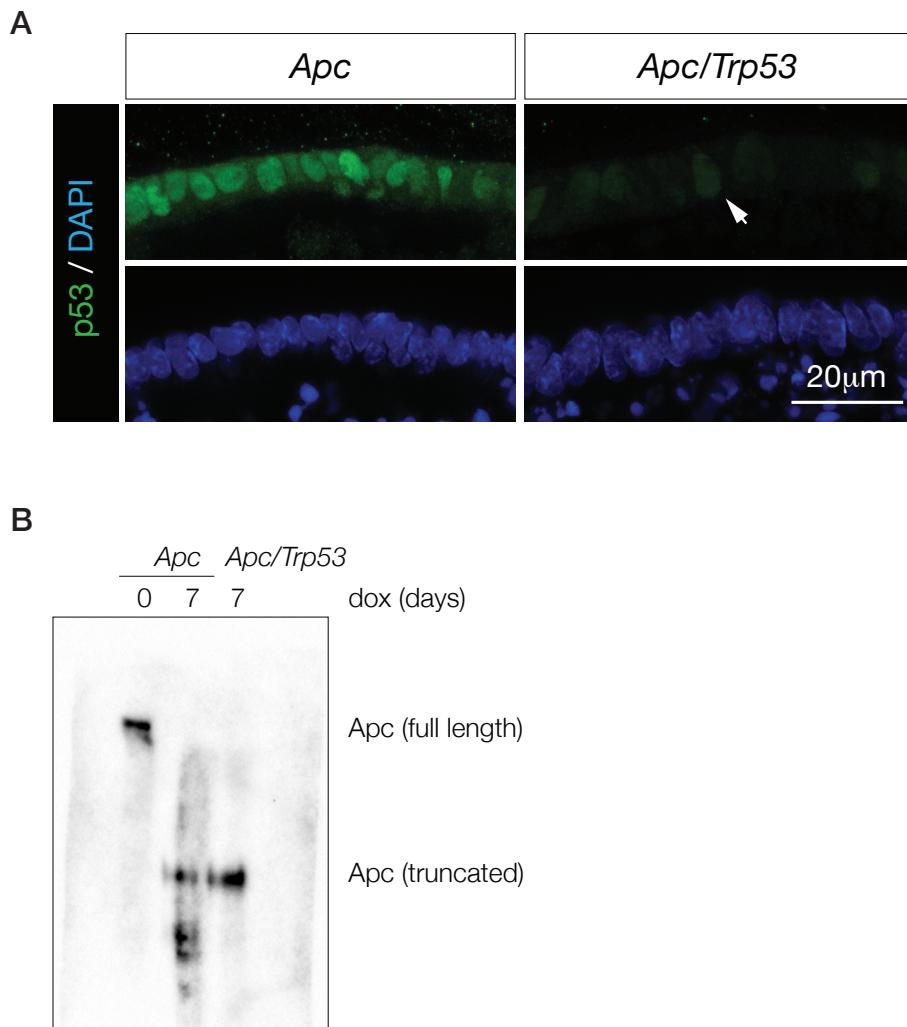
**Supplementary Figure 1:** Testing single and tandem sgRNAs with Cas9 and Cas9n. **A.** Surveyor assay on transfected ESC populations with Cas9 (PX330) or Cas9n (PX335) containing expression plasmids. Cas9n enables efficient target cleavage only in the presence of paired sgRNAs. **B.** Surveyor assay on dox-treated c3GIC9-Apc/Trp53 targeted ES cells to examine predicted off-target sites. Only one predicted off-target (p53-OFT7) showed significant cleavage. We were unable to perform surveyor assay on the OFT9 predicted region. **C.** Schematic representation of the tandem Cas9n vector used to express paired sgRNAs. Surveyor assay of Trp53 and predicted off-target site for this sgRNA in several individual c3GIC9-Trp53 and c3GIC9n-Trp53 ES cell clones. Use of the Cas9D10A nickase variant (c3GIC9n) with paired p53 sgRNAs prevents off-target cutting while preserving on-target efficiency. **D.** Surveyor assay on c3GIC9n-Apc (Apcn) ES cell clones carrying paired sgRNAs. Asterisk indicates dox-independent low-level detectable mutations in Apc.

## Supplementary Figure 2



**Supplementary Figure 2:** Inducible genome editing in the intestine of adult mice. **A.** Surveyor assay on genomic DNA from single (no R26-rtTA) or double (R26-rtTA / c3GIC9) transgenic mice as indicated, treated with dox for 10 days. Released bands from the surveyor assay indicated mutation events at the target loci only in those animals carrying R26-rtTA and c3GIC9 alleles. **B.** Quantitative PCR gene expression analysis of small intestinal crypts/villi following 10 days of dox treatment. R26-rtTA / c3GIC9-Apc and R26-rtTA / c3GIC9n-Apc mice showed dramatic induction of Wnt target genes, Axin2, Lgr5 and cMyc. **C.** Immunohistochemical and immunofluorescent images of intestinal sections from 10-day dox-treated c3GIC9-CR8, Apc, Apc/Trp53 and c3GIC9n-Apc (Apc<sup>n</sup>) mice with no R26-rtTA allele. Sections were stained for markers of proliferation (Ki67-green), differentiation (K20 – red) and Paneth cells (Lysozyme – brown). In mice that are unable to induce expression of Cas9 (no rtTA), we saw no evidence of hyperplastic overgrowth, blocked differentiation or ectopic production of Paneth cells. Scale bars are 100μm.

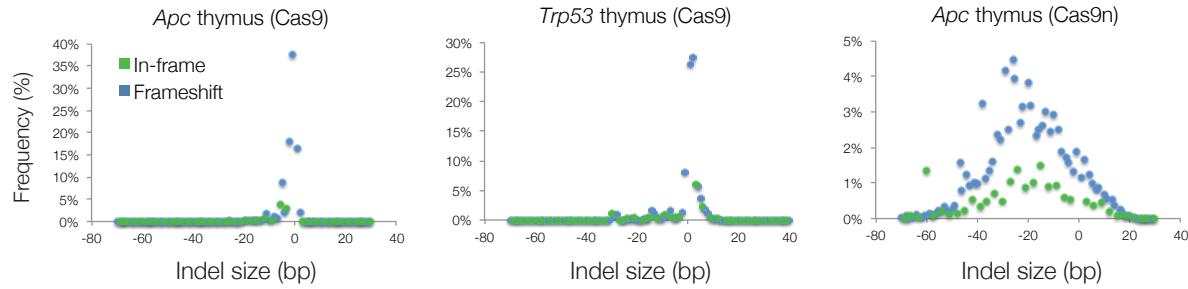
### Supplementary Figure 3



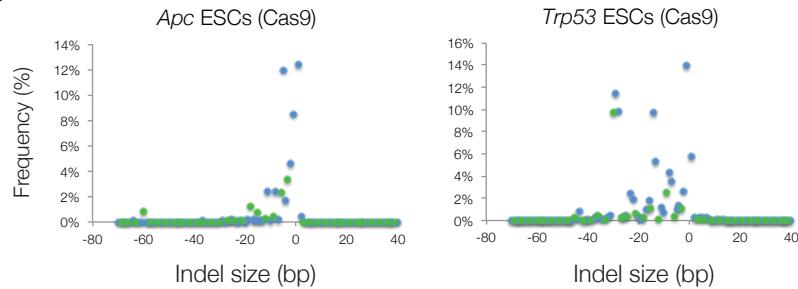
**Supplementary Figure 3:** **A.** p53 deletion in ex vivo organoid cultures. Immunofluorescent images of D7 dox-treated spheroids showing loss of p53 protein (green) in c3GCI9-Apc/Trp53 mice (right), but not c3GIC9n-Apc (left). Cultures were treated with 125ng/ml Adriamycin for 4hrs prior to staining, to induce expression of p53 protein. White arrow indicates low level p53 protein detectable in some cells within the structure. Scale bars are 20 $\mu$ m. **B.** Uncropped image of Apc western blot presented in Figure 2, showing presence of truncated Apc proteins following Cas9-mediated editing of Apc.

## Supplementary Figure 4

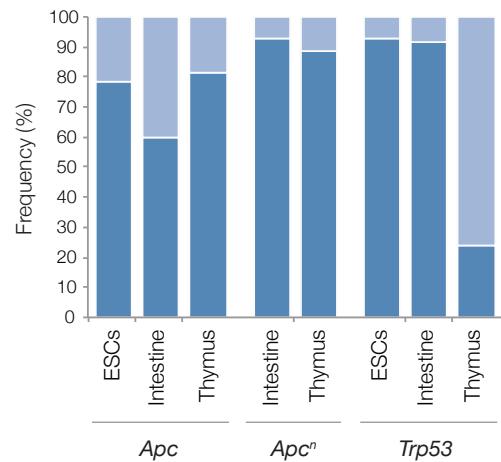
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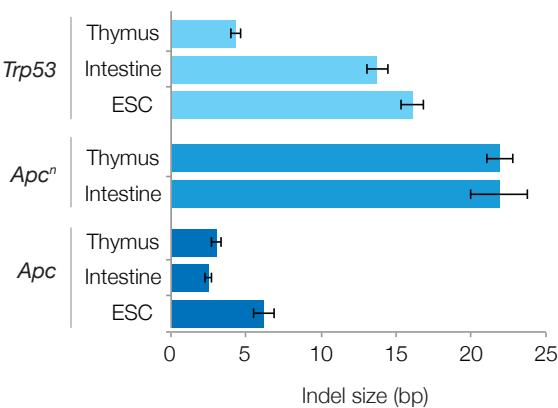
**B**



**C**

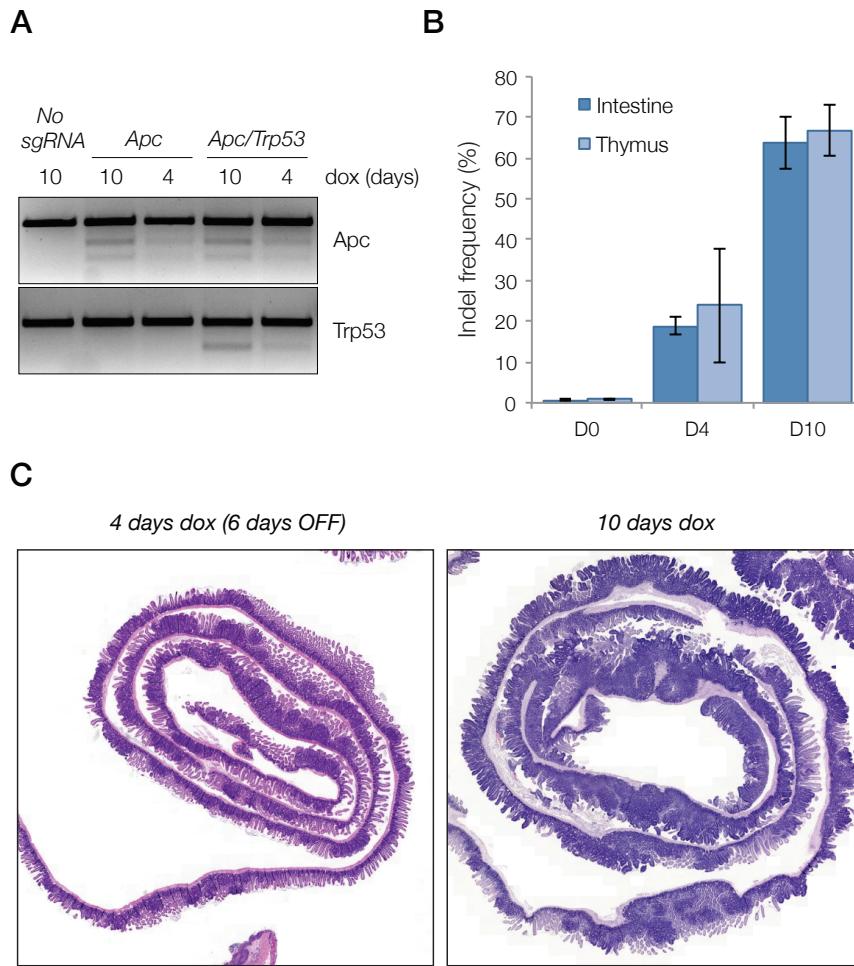


**D**



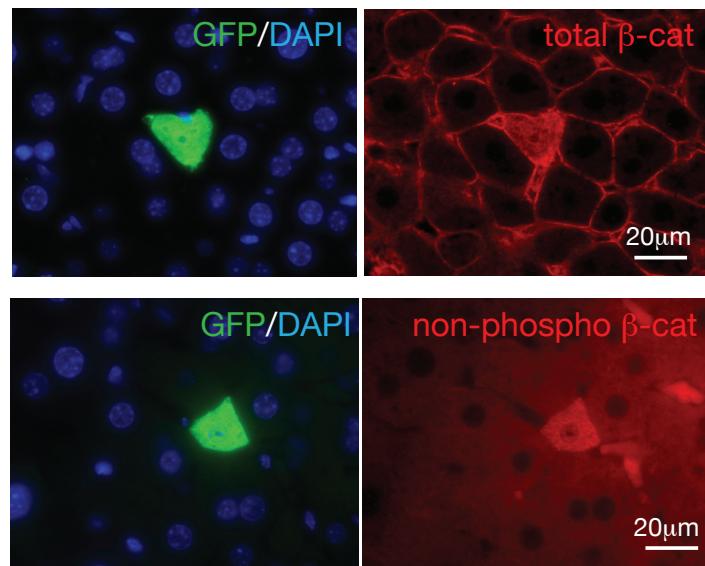
**Supplementary Figure 4:** Frequency and type of gene modifications following Cas9/Cas9n induction in mice. Scatter plots displaying frequency and size of indels in *Apc* and *Trp53* in dox-treated thymus (**A**) and ESCs (**B**) as indicated. Blue points indicate frameshift mutations while green points indicate in-frame indels. **C**. Frequency of insertions (light blue) and deletions (dark blue) in dox-treated intestine, thymus and ESCs for each target region in the presence of single (*Apc* and *Trp53*) or paired sgRNAs (*Apc<sup>n</sup>*). **D**. Size of indels in dox-treated intestine, thymus and ESCs for each target region in the presence of single (*Apc* and *Trp53*) or paired sgRNAs (*Apc<sup>n</sup>*). Error bars represent SEM,  $n \geq 3$ .

## Supplementary Figure 5



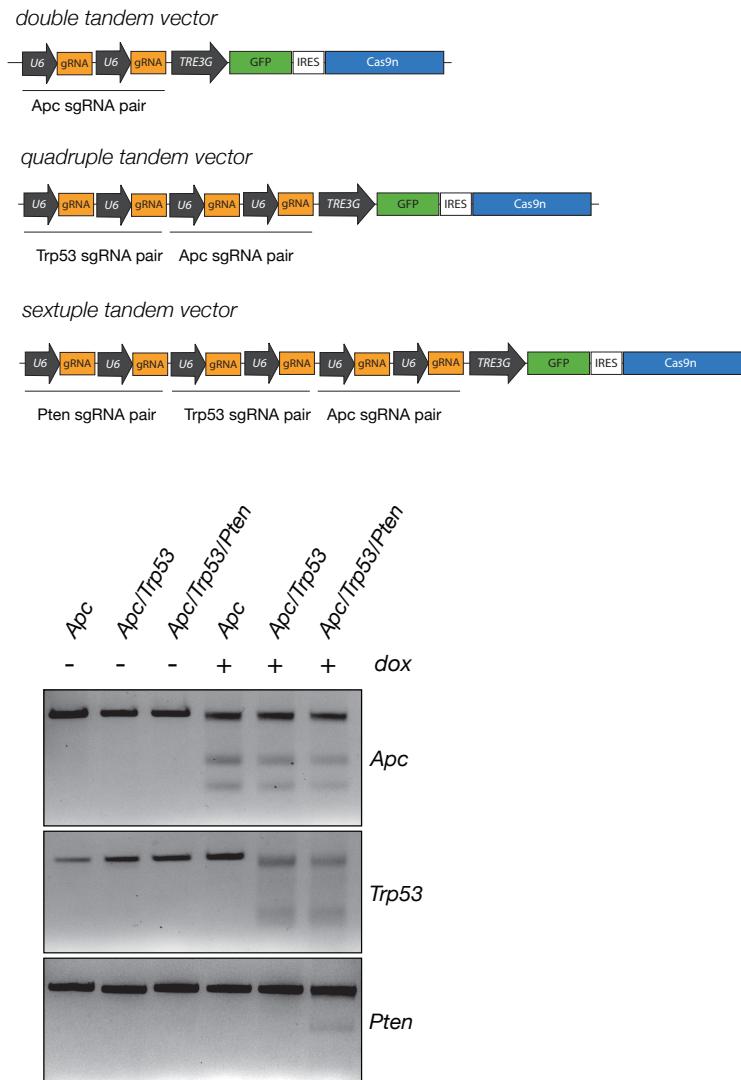
**Supplementary Figure 5:** Varying the time of Cas9 induction to control the frequency of target mutations. **A.** Surveyor assay on genomic DNA from R26-rtTA / c3GIC9-Apc and Apc/Trp53 transgenic mice treated with dox continually for 10 days, or pulsed with dox for 4 days and regular chow for 6 days. Transient expression of Cas9 was sufficient to induce mutational events at both Apc and Trp53 target sites, but at lower levels than those mice maintained on dox. **B.** Indel frequency assessed by deep sequencing the Apc locus in the intestine (dark blue) and thymus (light blue), in untreated, day 4 (D4) or day 10 (D10) dox-treated c3GIC9-Apc and c3GIC9n-Apc mice (Error bars represent SEM, n = 3). **C.** Representative images of D4 and D10 dox-treated c3GIC9-Apc intestine, 10 days following dox initiation, showing increased tumor burden in D10 treated animals.

## Supplementary Figure 6



**Supplementary Figure 6:** Mosaic and tissue specific induction of CRISPR/Cas9 in hepatocytes. Immunofluorescent images of CAGs-rtTA3 transfected c3GIC9n-Apc livers showing mosaic induction of GFP (linked to Cas9n) and corresponding increase in total (left) and non-phosphorylated (right)  $\beta$ -catenin, characteristic of Apc loss of function. Scale bars are 20 $\mu$ m.

## Supplementary Figure 7



**Supplementary Figure 7:** Targeting three genes from a single allele using tandem sgRNAs. Schematic representation of the tandem, quadruple and sextuple Cas9n targeting vector used to express one, two or three pairs of sgRNAs, targeting Apc, Trp53 and Pten. Surveyor assay of targeted ES cells showing modification of each targeted locus following 2 days of dox treatment.

**Supplementary Table 1**

Region	Matches	Pre-seed	Seed	PAM	Align	Chr	Strand	Forward primer	Reverse primer
APC_On target	23	8	12	3	xx      x	chr18	+	TACGGTATTGCCAGCTCTT	CGTCCTGGGAGGTATGAATG
APC_OFT1	19	5	11	3	x  xx    x   x	chr4	-	TCCCTTTCCATGTCCAATC	GCAGAGCTGAACAGTCCACA
APC_OFT2	19	5	11	3	xx  x   x   x	chr16	+	TGTGTGGATGAGACCCAGAA	TGGATCGTACACATGGCAGT
APC_OFT3	19	5	11	3	x x   x   x   x	chr19	-	CCTCGGTAGTGAGAGTTGG	GGACCCGATTCAAGTCACATC
APC_OFT4	18	4	11	3	xx xx     x   x	chr4	+	GAGTGCCTAGCCTCATGGAC	GAGGTCAAGAAGAGGGTGCCTG
APC_OFT5	17	3	11	3	xxxx   x   x   x	chr4	+	CACAGATTCAGCAGCCTTC	CTTCCCTGGCATAACCACGTA
APC_OFT6	17	3	11	3	xx xxx     x   x	chr5	+	TCCGAAGGACTGTTGAAGG	CCAATATGTCAGCCAGGTT
p53_OFT1	21	7	11	3	x     x   x   x	chr13	+	TTAAGTGGTGGCAGCTCCTT	AGTGCAAGCGGTTCAAGTTTC
p53_OFT2	19	5	11	3	x     x   x   x   x	chr14	-	GTTTACCCCTCGGAAGCTCAG	TGGGTTAGGACTTCCTGTGG
p53_OFT3	18	4	11	3	xxxx   x   x   x	chr16	+	GCTGTACATCATGCCAGCAA	GATGACCATCCCACACTCT
p53_OFT4	18	4	11	3	xx x   x   x   x	chr17	+	TCCCTCAGGATCTCACAG	CGGAAATGTGGATTCAAGGAT
p53_OFT5	19	5	11	3	x   xx     x   x	chr10	-	CGCTTGTCACTGTCGT	GGGAAAGGAGAAGGGACACT
p53_On target	23	8	12	3	x       x	chr11	+	TTTGAAAGGCCAACGTGAAG	CCACTCACCGTGCACATAAC
p53_OFT6	22	8	11	3	x       x	chr17	+	ATCCTGCCATCACCTCACTC	CCAGGTGGAAGCCATAGTTG
p53_OFT7	21	6	12	3	xx     x       x	chr10	+	ACACCCAGACAATGCCAAT	TGCTCTGAGAAGATGCTCCA
p53_OFT8	20	6	11	3	x x   x   x   x	chr17	+	GGGCTAGAGACCGAACGCC	ACTGCTAGCCACATGCCCT
p53_OFT9	18	4	11	3	x xx   x   x   x	chr8	+	ACCCAGAGGGCTTGTGCTA	CCTCCCTGGAGGGCTATCT
p53_OFT10	18	4	11	3	xxx   x   x   x	chrX	+	CTTCAGGCCTTCATCCCTCAG	CACTCCTGGAGACCAACTC
p53_OFT11	18	4	11	3	x xxx   x   x   x	chrX	-	CCCTCTCACACACTCTCT	TGGACATCCACTTGGCTCTT
p53_OFT12	17	3	11	3	xxxx   x   x   x	chr9	+	GAGGGTGTCTAGGGCACACA	CCTCCTGAGTACTGGGTCA

**Supplementary Table 2.** Mendelian transmission of *col1a1*-targeted CRISPR/Cas9 transgenes

Genotype	<i>CR8</i>	<i>Apc</i>	<i>Apc<sup>n</sup></i>	<i>Apc/Trp53</i>
Transgene transmission	31 (28.5) †	28 (28.5)	23 (20.5)	42 (45)
<i>p</i> -value (two-tail, binomial test)	0.597	0.999	0.5327	0.598
Het mutations in F1 progeny	2/31*	2/28*	1/23*	6/42 (Apc) & 5/42 (p53)*

† Numbers represent: observed (expected) from heterozygote x wildtype crosses

\* Indels were identical within each strain

### Supplementary Table 3

#### Complementary oligonucleotides used for cloning sgRNAs into pX330 and pX335

Apc- $\alpha$	CACCGCAGGAACCTCATCAAAACG	Oligo A
	AAACCGTTTGATGAGGTTCCCTGC	Oligo B
Apc- $\beta$	CACCGTGTGGATGGTAAGCACTG	Oligo A
	AAACCAGTCTTACCATCCAACAC	Oligo B
Trp53- $\alpha$	CACCGACCTGTACCGAGACCCC	Oligo A
	AAACGGGTCTCGGTGACAGGGTC	Oligo B
Trp53- $\beta$	CACCGAGGAGCTCCTGACACTCGG	Oligo A
	AAACCCGAGTGTCAAGGAGCTCCCTC	Oligo B
Pten- $\alpha$	CACCGACTTGTCTCCGCCGCGT	Oligo A
	AAACACGGCGGGAGGACAAGTC	Oligo B
Pten- $\beta$	CACCGCTCAGCCATTGCCTGTGTC	Oligo A
	AAACACACAGGCAATGGCTGAGC	Oligo B
CR8	CACCGACATTCTTCCCCACTGG	Oligo A
	AAACCCAGTGGGAAAGAAATGTC	Oligo B

#### PCRs for amplifying U6-sgRNAs from pX330 or pX335

U6-sgRNA-F (Nsil) ATGCTATGCATGAGGGCTATTCCCATGATT  
 U6-sgRNA-R (SbfI) TGACACCTGCAGGTCTAGCTCTAAAACAAAAAAGC

#### PCR primers for Surveyor assay

APC_SVR_F	GCCATCCCTTCACGTTAG	
APC_SVR_R	TTCCACTTGGCATAAGGC	
p53-SVR-F	CAGAAGATATCCTGGTAAGG	
p53-SVR-R	CTACAGGCTGAAGAGGAACC	
PTEN-ex7F	CTTAAGGATTCAAGATTGAAG	
PTEN-ex7R4	AATGAAGAGTCTGCCAATCT	(Primary) genomic amplification reverse primer
PTEN-ex7R	TAATTCTAACCAAAAGGCT	(Secondary) nested PCR reverse primer
Ch8-SVR-F	TAAGATGATTATCTGAATT CCTGGGG	
Ch8-SVR-R	TCTTATCCCCTGTGTTGGAA	

#### Quantitative PCR primers

Axin2_2702F	GCAGCTCAGCAAAAGGGAAAT
Axin2_2815R	TACATGGGAGCAGTGTCTCGT
cMyc_1262F	CTCAGTGGTCTTCCCTACCCG
cMyc_1611R	TGTCCAACTTGCCCTTGGC
Lgr5_QF	CAAGCCATGACCTTGGCCCTG
Lgr5_QR	TTTCCCAGGGAGTGGATTCTATT

#### PCR primers for MiSeq

APC_F	TACGGTATTGCCAGCTCTT
APC_R	CGTCCTGGGAGGTATGAATG
Trp53_F	TTTGAAAGGCCAAGTGAAG
Trp53_R	CCACTCACCGTGCACATAAC